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## ***SPRED1* germline mutations caused a neurofibromatosis type 1 overlapping phenotype**

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**KEY WORDS:** *SPRED1*; neurofibromatosis type 1-like syndrome; [Legius syndrome](#)

**2901 words**

## **ABSTRACT:**

**Objective:** Germline loss-of-function mutations in the *SPRED1* gene have recently been identified in patients fulfilling the National Institutes of Health (NIH) diagnostic criteria for neurofibromatosis type 1 (NF1) but with no *NF1* (neurofibromin 1) mutation found, suggesting a neurofibromatosis type 1-like syndrome.

**Methods:** 61 [index cases](#) with NF1 clinical diagnosis but no identifiable *NF1* mutation were screened for *SPRED1* mutation.

**Results:** We described one known *SPRED1* mutation (c.190C>T leading to p.Arg64Stop) and four novel mutations (c.637C>T leading to p.Gln213Stop, c.2T>C leading to p.Met1Thr, c.46C>T leading to p.Arg16Stop, and c.1048\_1060del leading to p.Gly350fs) in five French families. Their NF1-like phenotype was characterized by a high prevalence of café-au-lait spots, freckling, learning disability, and an absence of neurofibromas and Lisch nodules in agreement with the original description. However, we did not observe Noonan-like dysmorphism. It is noteworthy that one patient with the p.Arg16Stop mutation developed a monoblastic acute leukaemia.

**Conclusions:** In our series, *SPRED1* mutations occurred with a prevalence of 0.5% in NF1 patients and in 5% of NF1 patients displaying a NF1-like phenotype. *SPRED1* mutated patients did not display any specific dermatologic features that were not present in NF1 patients, except for the absence of neurofibromas that seem to be a specific clinical feature of NF1. The exact phenotypic spectrum and the putative complications of this NF1 overlapping syndrome, in particular haematological malignancies, remain to be further characterized. NIH diagnostic criteria for NF1 must be revised in view of this newly characterized [Legius syndrome](#) in order to establish a specific genetic counselling.

## INTRODUCTION

Germline mutations in genes involved in the Ras-mitogen activated protein kinase (MAPK) signalling pathway have been reported in several phenotypically overlapping syndromes with autosomal dominant transmission.[1] These observations provided a unifying mechanism -- Ras MAPK pathway constitutive activation -- leading to neuro-cardio-facial-cutaneous (NCFC) syndromes (Figure 1). These rare diseases include neurofibromatosis 1 (NF1, OMIM 162200), Noonan syndrome (NS, OMIM 163950), LEOPARD syndrome (LS, OMIM 151100), cardio-facio-cutaneous syndrome (CFC, OMIM 115150), and Costello syndrome (CS, OMIM 218040). Predisposition to malignancies is a known feature of NCFC syndromes with the exception of CFC.[2]

NF1 has a birth incidence of ~1/3000 and has been showed to be caused by autosomal dominant loss of function mutations of the *NF1* (neurofibromin 1; NM\_000267) gene in approximately 95% of patients fulfilling the National Institutes of Health (NIH) clinical diagnostic criteria.[3,4,5] *NF1* is located in 17q11.2 and contains 60 translated exons distributed over ~350 kb. Most of the *NF1* mutations (90 to 95%) are intragenic mutations (point mutation, splice mutation, small deletion, insertion, or duplication). The remaining *NF1* mutations (5 to 10%) are microdeletions encompassing the entire *NF1* gene and its neighbouring genes.[6] The protein encoded by *NF1* (neurofibromin) is a Ras-GTPase activating protein (Ras-GAP) that acts as a negative regulator of the Ras-MAPK cascade.[7]

Germline dominant loss-of-function mutations in the *SPRED1* (sprouty-related, EVH1 domain containing 1; NM\_152594) gene have recently been identified in five unrelated families and seven additional patients fulfilling the NIH diagnostic criteria for NF1 although no *NF1* mutation was found.[8] Remarkably, their phenotype referred as neurofibromatosis type 1-like syndrome (NFLS, OMIM 611431) consisted

of multiple café-au-lait spots, axillary freckling, and macrocephaly but without neurofibromas and Lisch nodules. The *SPRED1* gene, located in 15q13.2, contains seven coding exons, and encodes the *SPRED1* protein. Sprouty (*SPRY1*, -2, -3, and -4) and *SPRED* (*SPRED1*, -2, and -3) proteins are an evolutionarily conserved family of membrane-associated negative regulators of growth factor-induced ERK activation.[9,10] *SPRED1* specifically inhibits MAPK signalling by suppressing Raf phosphorylation and activation.[11]

In the present study, we confirmed the existence of loss-of-function mutations of *SPRED1* and the characterization of a NCFC syndrome with a NF1 overlapping phenotype. We identified three nonsense mutations, one missense mutation, and one frameshift deletion of *SPRED1* among 61 families fulfilling NIH diagnostic criteria for NF1 but with no identifiable *NF1* mutation. We then developed a molecular diagnostic algorithm for individuals displaying a NF1-like phenotype tailored to routine clinical practice.

## **PATIENTS, MATERIALS, AND METHODS**

### **Patients**

A database devoted to neurofibromatosis 1 has been established in France thanks to a grant from the French Clinical Research Program (coordinator: Pr. P. Wolkenstein, Henri Mondor Hospital, Créteil, France). The collection consists of 561 families, including 1697 individuals among which 1083 were NF1 patients fulfilling the NIH diagnostic criteria. The phenotype of each patient was described in a standardised way and was coupled with a comprehensive analysis of the *NF1* mutational spectrum including intragenic microsatellites polymorphisms study, *NF1* sequencing at both RNA and DNA levels, and real-time PCR-based gene dosage (data not shown).

Among the 561 NF1 [index cases](#), a *NF1* mutation was successfully identified in 512 [individuals](#). The 49 NF1 [index cases](#) lacking *NF1* mutations were selected as a first panel from this NF-France database to be screened for *SPRED1* mutation.

A second panel of 12 NF1 clinically diagnosed [index cases](#) with no *NF1* mutation was phenotypically selected from routine genetic counselling: their phenotypes lied within the NF1 spectrum but were distinctive, with a high prevalence of café-au-lait spots, axillary, and groin freckling but absence of neurofibromas and Lisch nodules.

All blood samples drawn for nucleic acids (DNAs and RNAs) extraction were collected after informed consent.

### **DNA and RNA extraction**

High-molecular-weight DNA was prepared by standard proteinase K digestion followed by phenol-chloroform extraction from whole-blood leukocytes. Total RNA was extracted from whole-blood leukocytes by using the acid-phenol guanidium method. The quality of the nucleic acids was determined by electrophoresis through agarose gels and staining with ethidium bromide (DNA and RNA) and the 18S and 28S RNA bands were visualized under ultraviolet light (RNA).

The somatic leukaemia DNA was extracted from bone marrow of patient II: 1 in Family 4 (showing 85% of blastic cells) upon diagnosis of acute leukaemia.

### ***SPRED1* mutation screening**

We performed *SPRED1* mutation screening at both RNA and genomic DNA levels. Mutational screening of *SPRED1* was carried out on RNA through the following procedure. After reverse transcription with Superscript II RNase H-Reverse Transcriptase (Invitrogen), two overlapping PCR fragments were generated by

Taqman PCR Core Reagent Kit (Applied Biosystems). Mutation screening was performed with the ABI BigDye terminator sequencing kit (Applied Biosystems) on an ABI Prism 3130 automatic DNA sequencer (Applied Biosystems). Sequences were aligned with Seqscape® analysis software (Applied Biosystems). The primer oligonucleotide sequences and PCR conditions are available on demand.

Genomic DNA was amplified with primers specific for *SPRED1* coding exons and their IVS boundaries. The primer oligonucleotide sequences and PCR conditions are available upon request. PCR was performed with the Taqman PCR Core Reagent Kit (Applied Biosystems). Mutation screening was performed using bidirectional DNA sequencing of purified PCR products.

### ***SPRED1* real-time PCR-based gene dosage**

In this method, quantitative values are obtained from the threshold cycle number at which the increase in the signal associated with exponential growth of PCR products begins to be detected by Applied Biosystems analysis software. The precise amount of genomic DNA added to each reaction mix (based on optical density) and its quality (i.e. lack of extensive degradation) are both difficult to assess. We therefore also quantified the *ALB* gene (encoding albumin and mapping to chromosome region 4q11-q13) as an endogenous DNA control, and each sample was normalized on the basis of its *ALB* content. The relative *SPRED1* copy number was also normalized to a calibrator, or 1X sample, consisting of genomic DNA from a normal subject. Final results, expressed as N-fold differences in the target gene copy number relative to the *ALB* gene and the calibrator, and termed "Ntarget", were determined as follows:  $N_{\text{target}} = 2^{(\Delta C_{\text{t sample}} - \Delta C_{\text{t calibrator}})}$ , where  $\Delta C_{\text{t}}$  values of the sample and calibrator are determined by subtracting the average Ct value of the target gene from the average



Ct value of the *ALB* gene. Given the target gene marker, samples with Ntarget values of 0.5 and 1.0 were considered deleted and normal, respectively. Primers for *ALB*, and *SPRED1* gene dosage were chosen with the assistance of the computer program Oligo 4.0 (National Biosciences). *SPRED1* exons 3, 4, and 8 were quantified to copy number. The primer oligonucleotide sequences and PCR conditions are available upon request. All PCR reactions were performed with an ABI Prism 7900 Sequence Detection System (Applied Biosystems) and the SYBR® Green PCR Core Reagents kit (Applied Biosystems). Experiments were performed in triplicates for each data point.

## RESULTS

### Germline DNAs molecular analysis

49 [index cases](#) with no identifiable *NF1* mutation from the French NF1 database were screened for mutation in the *SPRED1* gene. Two *SPRED1* heterozygous nonsense mutations were identified at both DNA and RNA levels: one known mutation (Family 1: c.190C>T leading to p.Arg64Stop) and one novel mutation (Family 2: c.637C>T leading to p.Gln213Stop) (Figure 2).

*SPRED1* mutational analysis of an additional set of 12 [index cases](#) selected from routine genetic counselling on the basis of their NF1-like phenotype (presence of café-au-lait spots and/or freckling and absence of neurofibroma and Lisch nodule), identified three novel heterozygous mutations: one missense mutation (Family 3: c.2T>C leading to p.Met1Thr), one nonsense mutation (Family 4: c.46C>T leading to p.Arg16Stop), and one frame-shift deletion (Family 5: c.1048\_1060del leading to p.Gly350fs) (Figure 2).

In families 1, 2, and 3, the *SPRED1* mutations were present in all affected individuals and were absent in three tested unaffected relatives (Family 1: individual II: 1, Family 2: I: 1, and Family 3: I: 1) (Table 1). In families 4 and 5 the proband showed *SPRED1* mutations.

No *SPRED1* deletion was found using real-time PCR-based gene dosage in any of the patients tested.

The clinical features of *SPRED1* tested individuals of families 1, 2, 3, 4, and 5 are displayed in Table 1.

### **Molecular analysis of the somatic leukemic cells of patient II: 2 (Family 4)**

In order to test the tumour-suppressor gene hypothesis, *SPRED1* molecular analysis was performed on the leukemic cells DNA of patient II: 2 of Family 4. No somatic additional *SPRED1* alteration (including point mutation and loss of heterozygosity) was observed.

## **DISCUSSION**

The purpose of this study was to confirm the recently identified loss-of-function mutations of *SPRED1* in a new NF1 overlapping syndrome [8] (named NFLS) and to specify its phenotype and incidence. We confirmed one known mutation (p.Arg64Stop) described in the original paper of Brems et al.[8] and reported four novel mutations (p.Gln213Stop, p.Met1Thr, p.Arg16Stop, and p.Gly350fs) in five French families fulfilling NIH NF1 diagnostic criteria. [Similar to](#) the mutation p.Gln215Stop previously reported by Brems et al.[8] the nonsense mutation p.Gln213Stop found in Family 2 also affected exon 7 of *SPRED1*. The missense mutation c.2T>C (leading to p.Met1Thr) identified at both DNA and RNA levels (ruling

**Table 1:** Patients clinical features and *SPRED1* analysis results. CAL, café-au-lait. U, unknown. M, male. F, female. -, negative. WT, Wild type for *SPRED1* mutational analysis.

| Subject  | SPRED1 analysis | Gender | Age bracket (years) | weight (kg) | Length (cm) | Head Circumference |              | CAL spots |          | Axillary freckling | Inguinal freckling | Neurofibromas, Lisch nodules, Optic glioma | Other   |
|----------|-----------------|--------|---------------------|-------------|-------------|--------------------|--------------|-----------|----------|--------------------|--------------------|--|---|
|          |                 |        |                     |             |             | (cm)               | (percentile) | < 1.5 cm  | > 1.5 cm |                    |                    |  |   |
| Family 1 |                 |        |                     |             |             |                    |              |           |          |                    |                    |  |   |
| I:2      | C.190C>T        | F      | [70-74]             | 78          | 147         | 57                 | 50-95        | 5         | 1        | unilat             | -                  | -  | One lipoma  |
| II:1     | WT              | F      | [40-44]             | U           | U           | U                  |              | -         | -        | -                  | -                  | -  |   |
| II:2     | C.190C>T        | M      | [40-44]             | 92          | 172         | 58.5               | 50-95        | 3         | 3        | -                  | unilat             | -  | Cephalalgia; learning disability  |
| II:4     | C.190C>T        | F      | [40-44]             | U           | 163         | 58                 | > 95         | 2         | 2        | bilat              | -                  | -  | One lipoma; Macrocephaly  |
| III:2    | C.190C>T        | M      | [10-14]             | 64          | 169         | 56.5               | 50-95        | 8         | 3        | bilat              | bilat              | -  | Cephalalgia   |
| III:3    | C.190C>T        | M      | [5-9]               | 22          | 115         | 52                 | 50           | 5         | 4        | -                  | unilat             | -  |   |
| Family 2 |                 |        |                     |             |             |                    |              |           |          |                    |                    |  |   |
| I:1      | WT              | F      | [60-64]             | U           | U           | U                  |              | -         | -        | -                  | -                  | -  |   |
| I:2      | c.637C>T        | M      | [55-59]             | 80          | 168         | 57                 | 50-95        | 2         | 5        | unilat             | unilat             | -  |   |
| II:2     | c.637C>T        | M      | [35-39]             | 76          | 170         | 54                 | 5-50         | 5         | 4        | -                  | -                  | -  |   |
| II:4     | c.637C>T        | M      | [30-34]             | 78          | 178         | U                  |              | 10        | 4        | bilat              | bilat              | -  |   |
| II:6     | c.637C>T        | M      | [30-34]             | 68          | 172         | 56                 | 5-50         | 5         | 4        | -                  | -                  | -  |   |
| II:7     | c.637C>T        | F      | [25-29]             | 55          | 165         | 55                 | 5-50         | 11        | 10       | bilat              | bilat              | -  |   |
| II:8     | c.637C>T        | M      | [10-14]             | 31          | 150         | 52                 | 5-50         | 7         | 7        | unilat             | bilat              | -  | Epilepsy  |
| III:1    | c.637C>T        | M      | [5-9]               | 19          | U           | 52                 | 5-50         | 5         | 4        | unilat             | unilat             | -  | Learning disability   |
| Family 3 |                 |        |                     |             |             |                    |              |           |          |                    |                    |  |   |
| I:1      | WT              | M      | [40-44]             | 90          | 170         | 58                 | 50-95        | -         | -        | -                  | -                  | -  | Body diffuse freckling  |
| I:2      | c.2T>C          | F      | [40-44]             | 67          | 144         | 53                 | 5-50         | 4         | 5        | unilat             | -                  | -  | Cephalalgia   |
| II:3     | c.2T>C          | F      | [10-14]             | 42          | 146         | 52                 | 5-50         | 10        | 10       | bilat              | bilat              | -  | Learning disability: dyslexia, dysgraphia, attention deficit; cephalalgia |
| II:4     | c.2T>C          | M      | [5-9]               | 23          | 124         | 52                 | 5-50         | 4         | 3        | -                  | -                  | -  | Diffuse freckling on face and back; weak speech and language delay        |
| Family 4 |                 |        |                     |             |             |                    |              |           |          |                    |                    |  |   |
| I:1      | c.46C>T         | M      | [35-39]             | 65          | 176         | 55                 | 5-50         | -         | 2        | -                  | -                  | -  |   |
| II:2     | c.46C>T         | M      | [0-4]               | 14          | 106         | 53                 | 50-95        | 13        | -        | -                  | -                  | -  | Monoblastic acute leukemia; Learning disability                           |
| Family 5 |                 |        |                     |             |             |                    |              |           |          |                    |                    |  |   |
| III:7    | c.1048_1060del  | F      | [35-39]             | 59          | 165         | U                  | U            | 15        | 19       | Diffuse freckling  |                    | -  |   |

out a putative RNA decay) at the initiation codon in exon 2 abolished initiation of translation at the normal start site. Although the first new potential AUG codon downstream of the mutation at the position corresponding to methionine 21 could conceivably be used as an in-frame initiation codon, the loss of 20 amino acids from the Ena/vasodilator-stimulated phosphoprotein (VASP) homology (EVH-1) N-terminal functional domain of **SPRED1** would probably result in a non-functional protein. It is notable that most of the reported mutations of **SPRED1** affect the EVH-1 domain.[8] Nonetheless; the precise consequences of the p.Met1Thr mutation at the protein level remain to be determined by experimental means. The frame-shift deletion p.Gly350fs affected the C-terminus SPROUTY-like (SPR) domain of **SPRED1**, like p.Ser383fs previously reported.[8] These five mutations may result in a loss of function of **SPRED1**, known to inhibit the Ras-MAPK cascade, consistent with the concept that NCFC syndromes are caused by aberrant activation of this pathway.

The mechanism of inheritance in the two large families 1 and 2 (with five and seven affected and mutated individuals respectively) was compatible with an autosomal dominant pattern with complete penetrance. The clinical features of patients with *SPRED1* defects in our study (Table 1) were quite similar to the ones described by Brems *et al.*[8] The phenotype lied within the NF1 spectrum but was distinctive, with a high prevalence of café-au-lait spots and axillary and groin freckling and an absence of neurofibromas and Lisch nodules. Remarkably, some previously additional reported features were also found in the reported patients: learning disability was observed in four of the five reported families (Families 1, 2, 3, and 4) and lipomas in one family (Family 2). However, macrocephaly was found in only one individual and Noonan-like dysmorphism was not observed in any of the five families (Table 1) contrary to the original description of Brems *et al.*[8] Based on these

observations, the designation “Neurofibromatosis 1-like phenotype” does not seem appropriate because of the absence of neurofibroma. We therefore propose to rename the condition caused by heterozygous *SPRED1* mutations as “Legius syndrome”. The café-au-lait spots and freckling found in *SPRED1* mutated patients in our study were similar to those found in NF1 patients with *NF1* mutations. Therefore *NF1* and *SPRED1* mutated patients could not be distinguished on the dermatologic features (aspect and number) of their café-au-lait spots and freckling. Moreover, learning disability seems to be a quite constant feature of this new syndrome. It is noteworthy that the behavioral deficits of *Spred1*<sup>-/-</sup> and *Spred1*<sup>+/-</sup> mice mimic the neurocognitive impairments in *Nf1*<sup>+/-</sup> mice with a remarkably similar synaptic phenotype. [12]

Among the 561 NF1 index cases of the first panel (fulfilling the NIH diagnostic criteria for NF1), 91% (512/561) showed a *NF1* mutation, 0.5% (2/561) showed a *SPRED1* mutation, and 8.5% (47/561) did not show mutation in neither *SPRED1* nor *NF1*. These data confirmed that *SPRED1* mutations are rare events that are likely to be underestimated because of their mild associated-phenotype that may cause less medical consultations compared to NF1 cases with neurofibromas. The NF1-like phenotype (here defined as café-au-lait spots and/or freckling with no neurofibroma and no Lisch nodule) had a poor prevalence among all NF1 cases: among the 561 NF1 index cases, 8% (43/561) displayed a NF1-like phenotype among which 79% (34/43) showed a *NF1* mutation, and 5% (2/43) showed a *SPRED1* mutation. Among the 49/561 (9%) NF1 index cases with no identifiable *NF1* mutation, 9/49 (18%) index cases showed an NF1 like phenotype: 6 were isolated NF1 cases (only one NF1 individual recruited per family) and 3 were families with at least two recruited NF1 individuals. Among these three families, only two showed at least one postpubertal

patient (neurofibromas usually develop in the late teens or early twenties). Interestingly, these two families were exactly those showing a *SPRED1* mutation (Family 1 and Family 2). In light of these new observations, we adapted our molecular diagnostic strategy to routine clinical practice: post pubertal patients with a family history of café-au-lait spots and/ or freckling with neither neurofibromas nor Lisch nodule are now screened for mutation in *SPRED1* before performing *NF1* screening. Families 3, 4, and 5 satisfied these conditions.

It is noteworthy that patient II: 2 in Family 4 developed a monoblastic acute leukaemia, suggesting a possible implication of *SPRED1* germline mutation. *SPRED1* is highly expressed in hematopoietic cells and negatively regulates haematopoiesis by suppressing SCF and IL-3 induced ERK activation.[13] Although rare, inherited predispositions to myeloid leukaemia have uncovered a critical role of hyperactive Ras signalling in normal myeloid growth and leukaemogenesis.[14-16] Moreover, individuals with NS, LEOPARD syndrome, and NF1 have a higher risk of haematological malignancies, including AML and the rare disorder juvenile myelomonocytic leukaemia (JMML).[2,17] With regards to these observations, *SPRED1* seemed to be a good candidate gene for leukaemia predisposition. However, molecular analysis of the tumour DNA did not show additional somatic *SPRED1* alteration (including point mutation and loss of heterozygosity), in contradiction to the Knudson two-hits model for tumour-suppressor genes. Considering our observation and the data implicating hyperactive Ras in the pathogenesis of JMML, as well as the role of *SPRED1* in modulating signals from hematopoietic growth factor receptors to Ras, it would be of interest to screen *SPRED1* for mutation in the ~20% of JMML cases showing no mutations in either

*KRAS*, *NRAS*, *PTPN11*, or *NF1* [2,18] so as to confirm its putative implication in leukaemogenesis.

In conclusion, *SPRED1* mutations were present in 0.5% of all NF1 cases and in 5% of NF1 patients displaying a NF1-like phenotype. *SPRED1* mutated patients did not display specific dermatologic features not present in NF1 patients, except for the absence of neurofibromas that seem to be a specific clinical feature of NF1. NIH diagnostic criteria for NF1 must now take into account these new data to actualize guidelines and to establish a specific genetic counselling. Further studies will be necessary to characterize the frequency of *SPRED1* mutations, the exact phenotypic spectrum (including learning disability), and the putative complications (in particular haematological malignancies) of this [Legius syndrome](#) caused by a new mechanism through which Ras-MAPK signalling is deregulated. These findings also urge to test families with NF1-like phenotype with no *NF1* and *SPRED1* mutations for additional genes involved in the Ras-MAPK pathway.

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## LEGENDS TO FIGURES

**Figure 1:** A schematic of Ras signaling. Germline mutations in key components of the Ras-mitogen activated protein kinase (MAPK) signalling pathway cause neuro-cardio-facial-cutaneous (NCFC) syndromes.

**Figure 2.** Pedigrees of families 1, 2, 3, 4, and 5. Squares and circles indicate males and females, respectively. Open symbols indicate unaffected individuals, filled symbols indicate affected individuals, arrows indicate proband, and symbols with a slash indicate deceased family members.

**LEOPARD Syndrome**

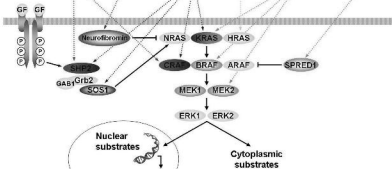
**NF1**

**Noonan Syndrome**

**Costello Syndrome**

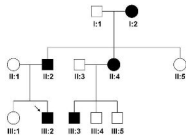
**CFC Syndrome**

**NF1-Like Syndrome**

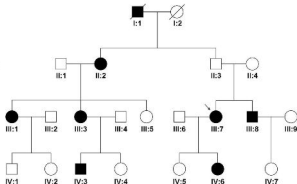


- Pathway interaction
- | Pathway inhibition
- .....→ Molecular alteration and NCFC syndromes

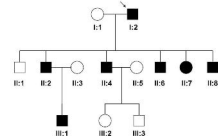
Family 1



Family 5



Family 2



Family 3



Family 4

